METHYLOTROPHIC BACTERIUM FOR THE PRODUCTION OF RECOMBINANT PROTEINS AND OTHER PRODUCTS

BACKGROUND OF THE INVENTION

(a) Field of the Invention

[0001] This invention relates to a system and method of producing recombinant peptides or proteins and other products from metabolic engineering in prokaryotes. More specifically, the present invention relates to the use of the methylotrophic bacterium *Methylobacterium extorquens* ATCC 55366, in combination with novel expression vectors, as an efficient expression system for recombinant peptides or proteins and industrially important bulk chemicals.

(b) Description of the Prior Art

[0002] Current microbial processes for the production of recombinant proteins use either eukaryotic microorganisms (e.g. *Pichia pastoris*), which may produce unwanted glycosylation and other unwanted post-translational modifications, or prokaryotic cells. The bacterium *Escherichia coli* is the best known and the most used prokaryotic expression system. The *E. coli* system, however, has drawbacks which include inclusion body formation (when undesired), high acetate production, which tends to inhibit growth and product formation, and a requirement for relatively expensive carbon sources such as glucose.

[0003] Methylotrophic bacteria are a group of prokaryotic microorganisms that can utilize one-carbon (C_1) compounds more reduced than carbon dioxide as a source of carbon and energy. Formaldehyde, an intermediate in the oxidation of reduced C_1 compounds, is incorporated into cells carbon via the serine pathway or

via other pathways, and/or can be further oxidized in a series of reactions to $C0_2$, generating energy in the form of reducing equivalents.

[0004] *Methylobacterium extorquens* ATCC 55366 is a pink pigmented facultative methylotroph isolated from a hydrocarbon-contaminated sandy soil [Bourque *et al.* (1992) Appl. Microbiol. Biotechnol. 37:7-12]. The growth of this bacterium in a fed-batch fermentation system developed by Bourque *et al.* [Bourque *et al.* (1995) Appl. Microbiol. Biotechnol. 44(3-4):367-376] resulted in cultivation at very high cell densities using a relatively cheap substrate, methanol, for the production of poly-β-hydroxybutyrate, a very interesting polyester.

[0005] The ability to produce high biomass densities in fermenters, combined with the newly acquired genetic information obtained from the genome sequencing of *M. extorquens* [Alper (1999) Science 283:1625-1626], renders this microorganism extremely interesting as a potential expression system for recombinant peptides or proteins and for the production of industrially important bulk chemicals. In order to achieve these objectives, it is essential to identify efficient cloning vectors and promoters for introducting new genes into *M. extorquens*.

[0006] It would be highly desirable to be provided with a method for the production of a large variety of products from metabolic engineering which would help overcome some of the current problems.

SUMMARY OF THE INVENTION

[0007] One aim of the present invention is to provide a method for the production of products from metabolic engineering which would help overcome some of the problems faced with current microbial processes.

[0008] Such methods would include a new non-pathogenic prokaryotic microbial system, as an alternative to *E. coli*, for recombinant peptide or protein expression which utilizes methanol as a source of carbon and energy for growth in chemically, protein-free, defined medium. Such methods would also include the use of the minimal culture medium combined with the use of methanol as a carbon and energy source, which would lower the costs of producing products from metabolic engineering. Such systems would also include a new prokaryotic microbial system capable of producing recombinant peptides or proteins and other products from metabolic engineering at levels comparable to *P. pastoris* (grams/litre) in a high cell density fermentation process from methanol. Such methods would further include the development of transformation vectors, which if not integrated into the host genome, then stably or satisfactorily maintained in the cells in the presence of selective pressure such as antibiotics.

[0009] In accordance with one preferred embodiment of the present invention there is provided A method of producing a recombinant peptide, a recombinant protein or a product from metabolic engineering using a genetically modified first methylotrophic bacterium under the control of a regulated promoter from a second methylotrophic microorgansim of the same or different species; comprising the steps of:

[0010] a) introducing into said first methylotrophic bacterium an expression vector comprising a polynucleotide sequence, encoding for a peptide or a protein or allowing production of a product from metabolic engineering, under the control of a regulated promoter;

[0011] b) growing said genetically modified first methylotrophic bacterium in a minimal salts medium lacking organic sugars and containing methanol for a time sufficient to allow production of said peptide or protein or said product from metabolic engineering; and

[0012] c) regulating expression of said polynucleotide sequence by said promoter.

[0013] In another preferred embodiment of the present invention the regulated promoter is a metal regulated promoter and step c) is effected with a metal ion.

[0014] In another preferred embodiment of the present invention the methylotrophic bacterium is of the species *Methylobacterium.*

[0015] In another preferred embodiment of the present invention the methylotrophic bacterium is Methylobacterium extorquens ATCC 55366.

[0016] In one preferred embodiment of the present invention the polynucleotide sequence is a gene encoding for green fluorescent protein.

[0017] In another preferred embodiment of the present invention the polynucleotide sequence is a gene encoding for an enzyme.

[0018] In another preferred embodiment of the present invention the polynucleotide sequence is a gene which encodes for a peptide or protein which is not an enzyme.

[0019] In one preferred embodiment of the present invention the enzyme reacts with a component within or from the culture medium to produce a peptide or protein, or other product from metabolic engineering.

[0020] In another preferred embodiment of the present invention the peptide or protein, or other product from metabolic engineering, reacts with a component within or from the culture medium to produce a product from metabolic engineering.

[0021] In one preferred embodiment of the present invention the polynucleotide sequence is inserted into a vector suitable for introduction into a methylotrophic bacterium, wherein the vector is stably maintained within the methylotrophic bacterium during growth and replication of the methylotrophic bacterium in the presence of selection pressure, and wherein the vector allows for the expression of the polynucleotide sequence within the methylotrophic bacterium.

[0022] In one preferred embodiment of the present invention the selective pressure is an antibiotic.

[0023] In one preferred embodiment of the present invention the regulating expression of the polynucleotide sequence by the promoter is with Cu.

[0024] In one preferred embodiment of the present invention the promoter is the promoter present in the soluble methane monooxygenase (sMMO) operon of *Methylosinus trichosporium* OB3b.

[0025] In another preferred embodiment of the present invention the promoter is a promoter from a gene from a methylotrophic bacterium.

[0026] In another preferred embodiment of the present invention the promoter is a promoter from a gene from an organism other than a methylotrophic microorganism.

[0027] In one preferred embodiment of the present invention the expression vector is p*mmoX*-GFP-pRK310.

[0028] In another preferred embodiment of the present invention the expression vector is p*mmoX*-GFP-pVK101.

[0029] In another preferred embodiment of the present invention the expression vector is p*Lac*-GFP-pJB3KmD

[0030] In another preferred embodiment of the present invention the expression vector is p*Lac*-GFP-pRK310.

[0031] In one preferred embodiment of the present invention the use can be for high-throughput peptide or protein production, or high-throughput production of other products from metabolic engineering.

[0032] In another preferred embodiment of the present invention the use can be for proteomics-based peptide or protein expression or proteomics-based expression of other products from metabolic engineering.

[0033] In one preferred embodiment of the present invention the growing the genetically modified methylotrophic bacterium is performed within a flask.

[0034] In another preferred embodiment of the present invention the growing the genetically modified methylotrophic bacterium is performed within a fermenter.

[0035] For the purpose of the present invention the following terms are defined below.

[0036] The term "methylotrophic bacterium" is intended to mean a group of prokaryotic microorganisms that can utilize one-carbon (C_1) compounds more reduced than carbon dioxide as a source of carbon and energy.

[0037] The term "GFP" is intended to mean green fluorescent protein.

[0038] The term "expression vector" is intended to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and may optionally include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

[0039] The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator.

[0040] The term "polynucleotide" denotes a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter

two terms may describe polynucleotides that are single-stranded or double stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base paris". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

[0041] The term "polypeptide" is intended to denote a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

[0042] The term "promoter" is intended to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

[0043] The term "facultative methylotroph" is intended to denote a bacterium able but not obliged to grow in and perhaps use methanol as a carbon and/or energy source, but will also survive and perhaps grow in the absence of methanol.

[0044] The term "obligate methylotroph" is intended to denote a bacterium obliged to grow in and perhaps use methanol as a carbon and/or energy source, the bacterium will not survive or grow in the absence of methanol.

[0045] The term "and other products from metabolic engineering" is intended to mean, without limitation, plasmids for gene therapy or to support R&D activities, enzymes (cellulases, proteases, lipases), pigments (beta-carotene, food colorants, anti-oxidants), vitamins (vitamin B12, biotin, riboflavin), amino acids (lysine, tryptophane, tyrosine, alanine), polysaccharides (pullulan, cellulose, chitin), biosurfactants (rhamnolipids, emulsan), biopesticides (Bt toxins, TMOF), hormones (insulin), antibiotics (tetracyclins, penicillins, gramicidin, kanamycin,), and biomaterials (silk, elastin, albumins).

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] Fig. 1 illustrates the schematic strategy used to create different GFP-carrying plasmids.

[0047] Fig. 2 illustrates GFP production by *M. extorquens* in LB or CHOI media.

[0048] Fig. 3 illustrates GFP production by *M. extorquens* dependent on [Cu] in the medium. Bars represent the error deveiation within four independent fluorescence measurements.

[0049] Fig. 4 illustrates the production of GFP during the growth of *M. extorquens* (clone 3-63, p*mmoX*-GFP-pVK101 construct). Bars represent the error deviation within four independent fluorescence measurements.

DETAILED DESCRIPTION OF THE INVENTION

[0050] The present invention relates to the use of a new prokaryotic expression system which can overcome drawbacks inherent in using current eukaryotic or prokaryotic cells for the production of recombinant peptides or proteins and other

products from metabolic engineering. In particular, the present invention relates to the use of various expression vectors which can be used for recombinant peptide or protein expression and production of other products from metabolic engineering in *M. extorquens*. *M. extorquens* is a prokaryotic methylotrophic bacterium known to lead to high biomass densities in fermenters and whose genome has been completely sequenced. This microorganism is, therefore, extremely attractive as a potential expression system. The present invention provides a new prokaryotic microbial system capable of producing recombinant peptides or proteins and other products from metabolic engineering at high levels in a high cell density fermentation process from methanol.

Bacterial strains, plasmids and growth conditions

[0051] *M. extorquens* ATCC 55366 [Bourque *et al.* (1992) Appl. Microbiol. Biotechnol. 37:7-12] was grown as described previously [Bourque *et al.* (1995) Appl. Microbiol. Biotechnol. 44(304):367-376]. The ATCC number 55366 is the number designated to the pruified specimen culture deposited on Oct. 14, 1992 with the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, Md. 20852, United States of America). Electro-competent cells of *M. extorquens* were prepared by the method of Toyama *et al.* [Toyama *et al.* (1998) FEMS Microbiol. Lett. 166(1):1-7] after slight modifications. Cells were grown in CHOI medium (containing 1% v/v methanol) until the culture reached an OD_{600} $\approx$ 0.6-0.8. Cells were harvested by centrifugation (1800 X g, 10 min, 4°C) and washed twice with ice-cold sterile 10% (v/v) glycerol solution. The cell suspension was concentrated 10-fold in 10% glycerol, dispensed in 400-µl

aliquots and kept at -80°C. Electro-competent cells (100 µl) were mixed with DNA solution (500 ng) in a 0.2-cm cuvette chilled on ice. Electroporation was carried out using a Gene Pulser (Bio-Rad) with the following parameters: 2.5 kV, 400 Ω, 25 µF, to a final field strength of 12.5 kV cm^{-1}. After cells had been pulsed, 1 ml of ice-cold sterile Luria-Bertani low salts (LBLS) medium was immediately added to the cuvette , the cell suspension transferred into a test tube, and then incubated at 30°C for 24 h. Transformed clones were selected in LBnS (Luria-Bertani without NaCl) agar medium with appropriate antibiotics (kanamycin, 50 µg ml^{-1}; tetracycline, 20 µg ml^{-1}). *Escherichia coli* DH5α (Life Technologies Gibco BRL) was cultivated at 37°C in LBLS broth or on agar plates. Plasmids in *E. coli* were selected with ampicillin (100 µg ml^{-1}), kanamycin (50 µg ml^{-1}) or tetracycline (20 µg ml^{-1}). Information on the plasmids used for the present invention is given in Table 1.

Construction of plasmids

[0052] *In vitro* DNA manipulation for cloning in *E. coli* was performed as described by Sambrook *et al.* [Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY]. The strategy used to create different GFP-carrying plasmids is represented in Fig. 1. The set of primers used were: (a) GFP/ *Bam*HL2 (5'-GAA TCG GGA TCC TCA GTT GTA CAG TTC ATC CAT GC-3'; *Bam*HI restriction site underlined) and RBS/*Pst*I.2 (5'-AAC AAA CTG CAG AAT AAT TTT GTT TAA CTT TAA GAA GG-3' ; *Pst*I restriction site underlined); and (b) RBS/*Mlu*I (5'-CAC GAC GCG TTG AAA TAA TTT TGT TTA ACT TTA AGA AGG-3', *Mlu*I restriction

site underlined) and GFP/*Xba*I (5'-TGC TCT AGA TCA GTT GTA CAG TTC ATC CAT GC-3', *Xba*I restriction site underlined). The polymerase chain reaction conditions in both cases were: hot start at 94°C for 2 min and then 30 cycles of amplification (94°C, 30 s; 55°C, 30 s; 72°C, 30 s) followed by a final extension at 72°C for 10 min.

Detection of GFP expression in *M. extorquens*

[0053] Selected clones of *M. extorquens* carrying GFP constructs were grown in LBnS or in CHOI medium containing 1% methanol and the appropriate antibiotic (kanamycin, 20 μg ml^{-1}; tetracycline, 20 μg ml^{-1}) at 30°C, 250 rpm. After 72 h of incubation, cells were harvested by centrifugation and washed twice with sterile, deionized water. Cells were resuspended in 700 μl of water and two 100 μl aliquots were dispensed into 96-well plates. The remaining 500 μl was used to determine cell dry weight. Cells harboring pJB3KmD, pRK310 or pVK101 were used as control, and their fluorescence was subtracted from values obtained with cells harboring plasmids containing the *gfp* gene.

[0054] GFP production was determined in *M. extorquens* cells growing in CHOI medium. Cells were initially grown in 50 ml CHOI medium until the end of exponential phase (OD_{600} = 0.8). A 2% inoculum was then used to start the growth curve in 200 ml CHOI medium during which samples were taken for measurement of OD_{600}, fluorescence and dry weight.

[0055] Fluorescence of cell suspensions was determined in a Cytofluor 2300 System (Millipore) under excitation and emission wavelengths of 485 and 530 nm, respectively.

Table 1

Plasmids used

Plasmid	Description	Source
pJB3KmD[a]	cloning vector, *lacZ', oriV, oriT*, Ap^r, Km^r, 6.1 kb	[1]
pRK310	cloning vector, *lacZ', oriV, oriT*, Tc^r, 19 kb	[2]
PVK101	cloning vector, *lacZ', oriV*, Tc^r , Km^r, 20 kb	[3]
PMTL1000	cloning vector, *lacZ', ori*, Ap^r, ~12 kb	[4]
pQB163	expression vector, *T7, ori*, Ap^r, 6.3 kb	[5]
GFP-pMTL1000	pMTL1000 with 0.78-kb insert containing GFP coding sequence from pQB163	This invention
P*Lac*-GFP-pJB3KmD	pKJ3KmD with 0.78-kb insert containing GFP coding sequence from pQB163	This invention
P*Lac*-GFP-pRK310	pRK310 with ~0.78-kb insert containing GFP coding sequence from pQB163	This invention
P*mmoX*-GFP-pRK310	pRK310 with ~9.5-kb insert containing sMMO and GFP coding sequence from pMTL1000	This invention
P*mmoX*-GFP-pVK101	pVK101 with ~9.5-kb insert containing sMMO and GFP coding sequence from pMTL1000	This invention

Ap^r, Km^r, Tc^r denote resistance to ampicillin, kanamycin and tetracyclin, respectively. [a]Accession Databank No. U75323.

[1] Blatny *et al.* (1997) Appl. Environ. Microbiol. 63(2):370-379.
[2] Toyama *et al.* (1998) FEMS Microbiol. Lett. 166-(1):1-7.
[3] Knauf and Nester (1982) Plasmid 8:45-54.
[4] Nielsen *et al.* (1997) Mol. Microbiol. 25(2):399-409.
[5] Quantum Biotechnologies, Inc. (1998) Autofluorescent Proteins: Applications Manual. 11NO98.

[0056] The concentration of GFP was calculated based on a linear relationship between concentration and fluorescence determined for solutions of purified GFP (Quantum Biotechnologies). GFP yield is reported as GFP concentration (μg) per unit of dry weight (g).

Determination of Cu concentration in solution

[0057] Cells grown in the presence of Cu were harvested by centrifugation and the supernatant fluid was collected for Cu analysis. Concentrated H_2SO_4 (93%) (0.5 ml) was added to 9.5 ml of supernatant fluid in order to maintain the pH lower than 2.0 (thus preventing Cu precipitation) for Cu ions analysis using inductively coupled plasma-atomic spectrometer (ICP-AS; Thermo Jarel Ash, Trace Scan). The result was corrected with the appropriate dilution factor and referred to as the final soluble Cu concentration. The total Cu concentration added to the medium at preparation was determined likewise by adding 5% (v/v) concentrated H_2SO_4 to the medium and then analyzing using ICP-AS.

Efficiency of transformation of *M. extorquens* by electroporation

[0058] An essential step in achieving the expression and stability of heterologous genes in methylotrophic hosts is through the use of suitable broad-host-range vectors. The plasmids used here, pJB3KmD, pRK310 and pVK101, are derived from broad-host-range vectors which were developed for Gram-negative bacteria [Knauf *et al.* (1982) Plasmid 8:45-54; Ditta *et al.* (1985) Plasmid 13:1349-153; Blatny *et al.* (1997) Appl. Environ. Microbiol. 63:370-379]. Table 2 shows the time constant values obtained , as well as the efficiency of transformation for the different constructs used to transform *M. extorquens*. An overall improvement was observed on the efficiency of transformation of each plasmid in the following order: pJB3KmD < pRK310 < pVK101. While the time constant values were within the range obtained by Toyama

et al. [Toyama *et al.* (1998) FEMS Microbiol. Lett. 166(1):1-7] when applying similar electroporation conditions, the efficiency of transformation observed in the present invention for pRK310 (~ 10^3 cells μg^{-1} DNA) was at least two orders of magnitude lower than that obtained by them. This value was, however, close to that obtained by Ueda *et al.* [Ueda *et al.* (1991) Ann. N. Y. Acad. Sci. 646:99-105] when they electroporated *M. extorquens* with pLA2917. Such differences might be due to strain differences or to diverse conditions used for preparing the electro-competent cells, as well as due to specific electroporation conditions such as the time constant produced after each energy discharge. Nonetheless, the transformation efficiencies obtained in the present invention are high enough for practical use in genetic manipulation. The cosmid pVK100 (which resembles pVK101 except for the presence of the *cos* factor in pVK100) was shown to be mobilized from *E. coli* strains into *M. extorquens* AMI (previously known as *Pseudomonas* sp. AM1) by conjugation at frequencies of 10^{-1} to 10^{-2} [Fulton *et al.* (1984) J. Bacteriol. 160(2):718-723]; these frequency values were lower than the ones observed in the present invention. There are very few reports in the literature on the use of electroporation as a means of introducing DNA into *M. extorquens*. Although conjugation has been the preferred technique for transforming methylotrophic bacteria, electroporation was proven here to be a faster and less laborious technique.

GFP production under the control of the *lacZ* promoter

[0059] The *gfp* gene used in the present invention originated from a modified construct of the wild-type GFP [Quantum Biotechnologies, Inc. (1998) Autofluorescent Proteins: Applications Manual. 11NO98]. Its transcription was under the regulation of the *lacZ* promoter or of the soluble monooxygenase gene

cluster promoter *mmoX* [Nielson *et al.* (1997) Mol. Microbiol. 25(2):399-409]. The *lacZ* promoter has been successfully used for the expression in GFP by several bacteria [Bermudez *et al.* (1999) Methods Enzymol. 302:285-295]. The fusion of *LacZ* regulative elements in constructs containing mosquitocidal endotoxins gene *(cryIVB)* from *Bacillus thuringiensis* led to a significant increase of *cryIVB* gene expression in the obligate methylotroph *Methylobacillus flagellatum* [Marchenko *et al.* (2000) J. Ind. Microbiol. Biotechnol. 24(1):14-18]. However, the absence of the *lacI* gene gives rise to a constitutive phenotype and thus the *lac* promoter is induced constitutively even without inducers [Park *et al.* (1999) J. Microbiol. Biotechnol. 9(6):811-819].

[0060] The *lac* promoter was recognized by *M. extorquens* in the present invention, in accordance with previous findings [Toyama *et al.* (1998) FEMS Microbiol. Lett. 166(1):1-7]. It was also found to be constitutively induced. An interesting observation arose from the comparison between the fluorescence produced by clones of *M. extorquens* carrying the GFP gene in either pJB3KmD or pRK310 (Fig. 2). The use of the latter led to at least a 100-fold improvement in the amount of GFP produced by each clone.

Table 2

Time constant produced and efficiency of transformation of *M. extorquens* by electroporation

Plasmid	Time constant	Efficiency of transformation
	(ms)'	(cells pg^{-1} DNA)
pJB3KmD	8.9	$1.2X\ 10^2$
pRK310	8.8	$2.3\ X\ 10^3$
pVK101	8.6	$1.1\ X\ 10^4$
P*Lac*-GFP-pJB3KmD	8.7	$0.8\ X\ 10^2$
P*Lac*-GFP-pRK310	8.8	$3.6\ X\ 10^3$
P*mmoX*-GFP-pRK310	8.7	$0.7\ X\ 10^2$
P*mmoX*-GFP-pVK101	8.1	$2.0X\ 10^3$

'Time of exposure of cells to the high field strength applied.

[0061] An important difference was also observed in the amount of GFP produced by clones growing in either LB or CHOI medium (Fig. 2). Independently of the construct used, an improvement of at least 30% in the yield of GFP was obtained when cells grew in CHOI medium. The CHOI medium was described as the ideal medium to obtain high biomass titers of *M. extorquens* [Bourque *et al.* (1995) Appl. Microbiol. Biotechnol. 44(3-4):367-376]. The hypothesis that nutritional limitations may interfere with the production of GFP or with its chromophore activity [Tsien (1998) Annu. Rev. Biochem. 67:509-544] should be further investigated in order to explain the significant difference in yields of GFP depending on the medium used.

GFP production under the control of *mmoX* promoter

[0062] In the present invention, *M. extorquens* was transformed with constructs containing the *gfp* gene under the control of the *mmoX* promoter present in the soluble methane monooxygenase (sMMO) operon of another methylotrophic

bacterium, *Methylosinus trichosporium* OB3b. In this microorganism, sMMO catalyzes the oxidation of methane to methanol. The transcriptional regulation of the *smmo* gene is known to be copper-dependent; sMMO is expressed only under conditions in which the copper-to-biomass ratio is low. This allows for a strict control of the expression of the gene under its promoter by controlling the concentration of Cu in the medium [Nielsen *et al.* (1997) Mol. Microbiol. 25(2):399-409].

[0063] Transformed *M. extorquens* cells were grown in defined medium (CHOI) so that the effect of varying the concentrations of Cu^{2+} on the production of GFP could be determined (Fig. 3). It was found that the promoter was recognized by *M. extorquens*, therefore, allowing for the expression of GFP. The increase in the initial Cu^2+ concentration (from 0 to 57 μM) did not interfere with cell growth (as observed by the final dry weight). However, expression of GFP by clones carrying both p*mmoX*-GFP-pRK310 and p*mmoX*-GFP-pVK101 was not strongly controlled by the presence of Cu in the medium and repression was observed to some extent when the initial concentration of Cu in the medium was increased up to 57 μM.

[0064] Copper speciation in the medium and its effect on the activity of sMMO is also relevant to understanding *smmo* regulation. Morton *et al.* [Morton *et al.* (2000) Appl. Environ. Microbiol. 66(4):1730-1733] found no detectable sMMO activity when > 2.63 μM Cu g $protein^{-1}$ was present. Moreover, different sMMO activities were observed, depending on the type of Cu complex present, which could be explained by the inability of cells to actively transport Cu complexes into the cells, thus reducing Cu bioavailability. In the present invention, analysis of the soluble Cu present in the medium at the end of the cell growth experiments revealed that between 11 and 21% of the Cu added to medium was present in its free form (Fig. 3). The

remainder Cu was probably precipitated as oxides, hydroxides or ligand complexes, or accumulated by the cells. While the effect of different species of Cu could affect the regulation of p*mmoX*, the final concentrations of free Cu in the medium should be high enough to totally inhibit p*mmoX* expression. However, a reduction of 41 and 33% in GFP production was observed when the final Cu concentration was 2.4 and 3.9 μM Cu mg biomass^{-1} (for the clones 1-3 and 3-63, respectively).

[0065] The results for growth of *M. extorquens* carrying the p*mmoX*-GFP-pVK101 construct (in the absence of Cu) (Fig. 4) showed that the yield of GFP during growth reached its maximum at mid-exponential phase (about 700 μg of GFP g biomass^{-1}) and decreased as the culture reached the early stationary phase of growth (350 μg of GFP g biomass^{-1} at stationary phase). Since the fluorescence of cells remained constant during the stationary phase, this suggested that the reduced GFP yield observed may be due to cessation of GFP production during this phase. There could be several factors related to the growth conditions of *M. extorquens* carrying p*mmoX*-GFP-pVK101 (including O_2 or redox potential limitations which are known to dramatically affect the maturation of GFP [Tsien (1998) Annu. Rev. Biochem. 67:509-544]) that could explain the apparent end or slowing down of GFP production during stationary phase.

[0066] GFP has now been used as a model heterologous protein in order to identify suitable vectors as well as efficient promoters for M. extorquens. pRK310 and pVK101 constructs containing Lac and mmoX promoters are valuable expression systems for GFP and the expression of other industrially more important genes in this bacterium should now be more easily accomplished.

[0067] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications

and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.